GROWTH IN SUCKLING-MOUSE BRAIN OF "IBV-LIKE" VIRUSES FROM PATIENTS WITH UPPER RESPIRATORY TRACT DISEASE

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With the use of human embryonic tracheal organ culture,[9] six strains of a medium-sized (120–160 mμ) virus bearing a close morphologic resemblance to avian infectious bronchitis virus (IBV) were recovered in this laboratory from patients with colds.[13] These viruses were detected when organ culture fluid harvests were concentrated ten times and examined with the electron microscope. In negatively stained preparations the viruses appeared round and moderately pleomorphic with widely spaced, club-shaped surface projections. The tentative designation of "IBV-like" viruses was given to members of this group, whose morphology clearly differed from that of the myxoviruses. The "IBV-like" viruses were shown to be inactivated by ether, but their growth was not inhibited by 5-bromodeoxyuridine.[12] Serologic studies in organ culture suggested that the patients from whom the viruses were recovered developed a neutralizing antibody response. However, further study of these viruses was impeded by the lack of a tissue culture or experimental animal system which would support their growth. For this reason, and because we found that mouse hepatitis virus (MHV), another RNA-containing ether-labile virus, was morphologically identical to members of the "IBV-like" virus group in negatively stained preparations,[2] newborn mice were inoculated with the "IBV-like" virus strains. Encephalitis occurred in mice inoculated with two of the six strains. The preliminary results of these studies are reported here.

Growth of "IBV-Like" Viruses in Suckling-Mouse Brain.—"IBV-like" viruses were originally recovered by inoculating throat washings from patients with colds onto human embryonic tracheal organ cultures. Suspensions used for mouse inoculation were prepared from material passaged four to five times in organ culture and contained "IBV-like" virus particles when examined by electron microscopy after tenfold concentration. Pregnant Swiss mice of the CD-1 strain were obtained from Charles River Mouse Farms, Inc., Wilmington, Mass. Several years previously, the mice from this colony had been shown to be free of MHV contamination.[16] During our studies we were unable to demonstrate the presence of MHV antibody or infection in mice at the time they were received in the laboratory. The mice were kept in battery jars with a tightly fitting pad of $^3/_8$-inch glass-fiber material inserted in the lid. Strict isolation precautions were observed, and the mice were housed in a room used only for these experiments.

Suckling mouse passages were performed either by combined intracranial (IC) and intraperitoneal (IP) inoculation or by the IC route alone. On first passage, two of the six strains, designated OC38 and OC43, caused a disease which began 11–15 days after inoculation and was characterized by generalized tremors, rigidity, and lethargy. Two of 8 mice inoculated with OC38, and 3 of 16 mice inoculated with OC43 were affected. At the same time 16 control uninoculated mice remained well. On serial subpassage of brain suspensions, the syndrome

developed after progressively shorter incubation periods. At the fourth passage all inoculated mice died, and in that and later passages the period between inoculation and death was stabilized at 48–60 hours. Control subpassages were carried out in parallel with the infected passage series. None of the control mice developed an encephalitic syndrome.

A preliminary histologic study of tissue from affected mice showed round-cell infiltration in the meninges and in the perivascular spaces of the cerebral cortex. However, there was no evidence of inflammation in the liver, heart, or lungs.

Ten per cent brain suspensions of sixth or seventh passage material contained $10^{4.5}$ to $10^{5.5}$ 50 per cent lethal doses (LD_{50}) per 0.03 ml when tested by the IC route in suckling mice. However, the same suspension, when tested by the IP route, contained only $10^{0.5}$ LD_{50} per 0.05 ml. Encephalitis was induced by IC inoculation of mice five days of age or younger, but weanling mice inoculated by either the IC or IP route did not develop signs of illness. Asymptomatic infection probably occurred in the latter group, since serum drawn three weeks following inoculation contained both neutralizing and complement-fixing (CF) antibodies.

Tenfold concentrated seventh-passage mouse brain suspension contained "IBV-like" virus particles when examined by electron microscopy using the negative staining technique. Moreover, mouse-adapted virus at all passage levels appeared to replicate in human embryonic tracheal organ cultures, as indicated by electron microscopic examination of culture harvests. Adaptation of one human organ culture-grown virus strain, OC43, to suckling-mouse brain was attempted a second time and successfully repeated. However, this adaptation differed from the first in that mice developed encephalitic signs only after two blind passages. Virus recovered from the brains of affected third passage mice was identical to the originally adapted virus strain in its serological reactivity.

Preparation of CF Antigen and Serologic Responses of Individuals Infected with "IBV-Like" Viruses.—Clarified 10 per cent suspensions of sixth or seventh passage suckling-mouse brain fixed complement in the presence of homologous human convalescent serum. Furthermore, the patients from whom strains OC38 and OC43 were recovered developed a rise in CF antibody to both strains (Table 1). In contrast, these individuals failed to develop a rise in CF antibody to strain 229E, a tissue-culture-propagated virus with an identical morphology[1, 15] recovered from patients with upper respiratory disease.[5] When tissue-culture-grown MHV, strain A-59, which was shown previously to fix complement with some human sera[7] was tested, a four fold CF antibody rise in both serum pairs was evident in three of five replicate tests. One further test, that shown in Table 1, was indeterminate. In those tests in which a CF antibody rise was not demonstrated a 25–50 per cent fixation of complement by convalescent sera was observed. However, such reactions are not considered positive by the arbitrary criteria used in our laboratory. The fixation of complement with MHV antigen appears to be very sensitive to the complement levels used in each individual test.[6] Mouse brain suspensions of MHV failed in all instances to fix complement with these sera. In contrast, a rise in CF antibody to both "IBV-like" virus strains was observed consistently in all tests. The CF data in Table 1 represent the results of a single test.

The patients who yielded the two "IBV-like" virus strains also developed rises in

neutralizing antibody to both virus strains, in most cases fourfold or greater (Table 1). IC inoculation of suckling mice and 27–47 LD_{50} of virus were employed in these tests. Using a plaque-reduction technique,[4] an antibody rise was not detected to strain 229E. Furthermore, neutralizing antibody to MHV, strain A-59, could not be demonstrated in either acute or convalescent sera when tests were performed in mouse liver tissue culture NCTC 1469,[8] using roller tubes and sixteen 50 per cent tissue-culture-infective doses ($TCID_{50}$) of virus.

Antigenic Relationships of "IBV-Like" Viruses.—Serum drawn three weeks following a single IC inoculation of OC38 or OC43 into 3- to 4-week-old weanling mice was used to compare the two "IBV-like" virus strains. In suckling-mouse cross-neutralization tests and in CF tests the two strains appeared serologically identical.

Hyperimmune sera were prepared in weanling mice by administering a second IC injection three weeks after the first and bleeding the mice one week later.

TABLE 1

SEROLOGIC REACTIONS OF SERA FROM PATIENTS YIELDING "IBV-LIKE" VIRUSES

		Reciprocal of Serum Antibody Titer against Indicated Agent							
		Complement Fixation[a]				Neutralization[b]			
Agent recovered (strain)	Serum	OC38	OC43	Strain 229E	MHV (A-59)	OC38[c]	OC43[c]	Strain 229E[d]	MHV (A-59)[e]
OC38	Acute	<4	<4	<4	<4	64	32	256	<8
	Convalescent	8	16	<4	4	256	100	256	<8
OC43	Acute	<4	<4	4	<4	64	25	64	<8
	Convalescent	16	16	4	4	256	200	64	<8

[a] Performed by the microtiter technique using 1.7 units of complement and overnight fixation at 4°–8°C.[17] Control tissue antigens showed no fixation of complement with these sera.

[b] Plaque-neutralization antibody titers measured at 60% reduction. Other titers calculated by the method of Reed and Muench.[15]

[c] Test performed in suckling mice using IC inoculation and 27–47 LD_{50} of virus. Sera in all neutralization tests were inactivated for 30 min at 56°C.

[d] Plaque-reduction technique, using human diploid fibroblast (WI-38) Petri dish cultures.[4]

[e] Test performed in NCTC 1469 roller-tube-cultures using 16 $TCID_{50}$ of virus.[8]

These sera contained high titers of neutralizing antibody ($\leq$1:2560 vs. 100–115 LD_{50} of virus in suckling mice) to both "IBV-like" virus strains (Table 2). The sera failed at the lowest dilution tested to neutralize strain 229E, MHV (strain A-59), or IBV (Beaudette 42 strain). Similarly, guinea pig hyperimmune serum against strain 229E and chicken hyperimmune serum against IBV (Mass. 41 strain) failed to neutralize the two "IBV-like" viruses in suckling mice. MHV, on the other hand, showed a consistent one-way serologic relationship with "IBV-like" viruses. High-titered polyvalent MHV antiserum (prepared against strains A-59,[11] MHV-1,[10] JHM,[3] and MHV-S[16]) contained low but consistently detectable levels (1:10–1:25) of neutralizing antibody to both "IBV-like" virus strains. Table 2 shows several further neutralization tests between members of this virus group. It is clear from these data that strain 229E, unlike the "IBV-like" viruses, is not serologically related to MHV.

A similar pattern emerged when these relationships were examined by the CF technique. Mouse hyperimmune sera against the "IBV-like" viruses contained moderately high levels of CF antibody (1:80–1:320 vs. 4–8 units of antigen) to both "IBV-like" virus strains. In contrast, CF antibody to strain 229E or to

any of five strains of MHV (A-59, MHV-1, JHM MHV-S, and MHV-11[14]) was not detected in these sera. IBV was not tested, since we have not yet developed a CF system for this virus.

Similarly, no rise in CF antibody to the "IBV-like" viruses was observed in three pairs of acute and convalescent sera which showed a fourfold or greater rise in CF antibody to strain 229E. These serum pairs, one of which was kindly supplied by Dr. D. Hamre, were obtained from patients who had each yielded a virus antigenically indistinguishable from strain 229E. On the other hand, monovalent mouse immune sera prepared against each of four strains of MHV (A-59, MHV-1, JHM, and MHV-S) fixed complement in the presence of the "IBV-like" virus antigens. These reactions were consistently two- to fourfold lower than those seen with the homologous virus strain. A more extensive discussion of the serologic relationships among this morphologically similar group of viruses will be published.

TABLE 2

SEROLOGICAL RELATIONSHIPS AMONG MORPHOLOGICALLY SIMILAR VIRUSES AS EXAMINED BY NEUTRALIZATION

Virus, strain	Test system	Virus inoculum used in tests	Reciprocal of Neutralizing Antibody Titer in Antiserum Prepared against Indicated Virus[a]: "IBV-Like" OC38 (mouse)	"IBV-Like" OC43 (mouse)	229E (guinea pig)[b]	MHV polyvalent (mouse)[c]	IBV Mass. 41 (chicken)[d]
"IBV-like" OC38	Suckling mice	32–100 LD_{50}	>2560	>2560	<10	25	<10
"IBV-like" OC43	Suckling mice	70–115 LD_{50}	2560	2560	<10	10	<10
Strain 229E	Human diploid fibroblasts	Plaque reduction[4]	<10	<10	250	<10	<10
MHV, A-59	Mouse liver roller tube cultures[e]	16–30 $TCID_{50}$	<10	<10	<10	2560	<10
IBV, Beaudette 42	Embryonated eggs[f]	3 EID_{50}[g]	<10	<10	ND[h]	ND[h]	>1280

[a] Sera were inactivated at 56°C for 30 min. Virus-serum mixtures were incubated for 2 hr at room temperature before inoculation. Antibody and virus titers were calculated by the method of Reed and Muench.[15]
[b] Prepared by repeated intraperitoneal inoculation of virus grown in human diploid fibroblasts.[2]
[c] Prepared against strains A-59, MHV-1, JHM, and MHV-S.
[d] Kindly supplied by Dr. Charles H. Cunningham.
[e] Tissue culture line NCTC 1469.
[f] Allantoic inoculation of 10-day-old embryonated eggs from Duckworth Hatchery and Feed Co., Hanover, Md.
[g] 50% egg-infective doses.
[h] Not done.

Differentiation of "IBV-Like" Viruses Propagated in Mouse Brain from Mouse Hepatitis Virus.—In view of the extensive prevalence of MHV in mouse colonies,[16] the mouse-adapted "IBV-like" virus strains must be clearly differentiated from known strains of MHV. As mentioned previously, the adaptation of organ-culture-grown "IBV-like" viruses to suckling mice could be repeated, with the recovery from mouse brain suspensions of virus which reacted with specific hyperimmune serum. Mice in all control passages failed to yield virus or to develop encephalitis. High-titer immune sera to both "IBV-like" virus strains failed at low dilutions to fix complement with MHV antigens. This is in contrast to the known MHV strains whose immune sera cross-react widely with other MHV strains. In addition, all known strains of MHV cause hepatitis with focal infiltration of the liver. We did not see hepatic lesions in mice inoculated with "IBV-

like" viruses. Moreover, we have been unsuccessful to date in demonstrating growth of MHV, strain A-59, in human embryonic tracheal organ culture.

Additional evidence that one of the mouse-adapted viruses (strain OC43) was serologically similar to its organ-culture-passaged counterpart was provided by a neutralization test in organ culture, performed by a technique reported previously.[13] The suspension of virus strain OC43 used in this test had been passaged previously only in human embryonic tracheal organ culture. Its growth in organ culture, as determined by the appearance in the culture medium of particles with characteristic electron microscopic morphology, was inhibited by a 1:640 dilution of mouse antiserum prepared against mouse-adapted virus strain OC43 (homologous titer 1:2560 in suckling mice), but not by a 1:40 dilution (the lowest tested) of MHV polyvalent mouse antiserum (homologous titer 1:2560 in roller tube cultures).

Use of "IBV-Like" Virus CF Antigen Derived from Mouse Brain for Epidemiologic Survey.—The CF antigens described in this report may represent a valuable tool for the serologic study of the epidemiology of "IBV-like" virus infection in man. Previously we reported the recovery of "IBV-like" viruses from five of nine patients who experienced colds during the winter of 1965–1966, a period when rhinovirus infection was uncommon.[13] Although the number of individuals studied was small, this finding suggested that these viruses were commonly associated with upper respiratory tract disease during the study period. To assess further the frequency of infection with these agents during the winter of 1965–1966, paired sera from 59 consecutive patients (including the 9 studied by the organ culture virus recovery technique) were tested by the CF method using mouse brain antigen. Eighteen of these individuals (including the 5 from whom "IBV-like" viruses were recovered) developed a fourfold or greater rise in CF antibody to "IBV-like" virus antigen. A more extensive report on the epidemiology of infection with "IBV-like" viruses will be published.

Summary.—Six viruses recovered from patients with upper respiratory tract disease and bearing a close morphologic resemblance to both infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV) were inoculated into suckling mice. Two of the six "IBV-like" strains grew in mice and caused an encephalitic syndrome. Brain suspensions from affected mice fixed complement with homologous human convalescent sera and specific mouse immune sera. In neutralization and complement-fixation tests the two strains were shown to be identical with each other and distinct from IBV and strain 229E (another human respiratory virus morphologically similar to IBV). However, they showed a consistent one-way serologic relationship with several strains of MHV. The evidence that these mouse-adapted "IBV-like" viruses are distinct from known strains of MHV was presented. In a preliminary seroepidemiologic survey, approximately one third of patients with common colds during the winter of 1965–1966 developed complement-fixing antibody for the "IBV-like" virus antigens.

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[1] Almeida, J. D., and D. A. J. Tyrrell, *J. Gen. Virol.*, **1**, 175 (1967).

[2] Becker, W. B., K. McIntosh, J. H. Dees, and R. M. Chanock, *J. Virol.*, **1**, 1019 (1967).

[3] Cheever, F. S., J. B. Daniels, A. M. Pappenheimer, and O. T. Bailey, *J. Exptl. Med.*, **90**, 181 (1949).

[4] Hamre, D., D. A. Kindig, and J. Mann, *J. Virol.*, **1**, 810 (1967).

[5] Hamre, D., and J. J. Procknow, *Proc. Soc. Exptl. Biol. Med.*, **121**, 190 (1966).

[6] Hartley, J. W., personal communication.

[7] Hartley, J. W., W. P. Rowe, H. H. Bloom, and H. C. Turner, *Proc. Soc. Exptl. Biol. Med.*, **115**, 414 (1964).

[8] Hobbs, G. L., K. K. Sanford, V. J. Evans, and W. R. Earle, *J. Natl. Cancer Inst.*, **18**, 701 (1957).

[9] Hoorn, B., and D. A. J. Tyrrell, *Brit. J. Exptl. Pathol.*, **46**, 109 (1965).

[10] Gledhill, A. W., and C. H. Andrewes, *Brit. J. Exptl. Pathol.*, **32**, 559 (1951).

[11] Manaker, R. A., C. V. Piczak, A. A. Miller, and M. F. Stanton, *J. Natl. Cancer Inst.*, **27**, 29 (1961).

[12] McIntosh, K., unpublished.

[13] McIntosh, K., J. H. Dees, W. B. Becker, A. Z. Kapikian, and R. M. Chanock, these PROCEEDINGS, **57**, 933 (1967).

[14] Morris, J. A., J. C. Parker, and S. S. Cross, *Federation Proc. (Abstracts)*, **25**, 727 (1966).

[15] Reed, L. J., and H. Muench, *Am. J. Hyg.*, **27**, 493 (1938).

[16] Rowe, W. P., J. W. Hartley, and W. I. Capps, *Proc. Soc. Exptl. Biol. Med.*, **112**, 161 (1963).

[17] Sever, J. L., *J. Immunol.*, **88**, 320 (1962).